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ARTICLE

Two-dimensional Infrared Spectroscopy: An emerging analytical tool?

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Ultrafast two-dimensional infrared (2D-IR) spectroscopy has provided valuable insights into biomolecular structure and dynamics, but recent progress in laser technology and data analysis methods have demonstrated the potential for high throughput 2D-IR measurements and analytical applications. Using 2D-IR as an analytical tool requires a different approach to data collection and analysis compared to pure research applications however and, in this review, we highlight progress towards usage of 2D-IR spectroscopy in areas relevant to biomedical, pharmaceutical and analytical molecular science. We summarise the technical and methodological advances made to date and discuss the challenges that still face 2D-IR spectroscopy as it attempts to transition from the state-of-the-art laser laboratory to the standard suite of analytical tools.

Introduction

The suite of analytical tools available to the modern chemist is substantial in both number and range of capability. The ability to visualise molecular structures comes via crystallography, nuclear magnetic resonance (NMR) or optical spectroscopies. Molecular masses are measured by high resolution mass spectrometry with a range of ionization methodologies that extend the technique to previously inaccessible categories of sample. Calorimetry techniques measure ligand binding affinity, separation technologies include liquid and gas-phase chromatography. Imaging tools at increasing resolution allow topographical analysis of materials and can be combined with spectroscopy for site specific chemical insight, while new techniques such as electron cryo-microscopy look set to revolutionise how biomolecular structures are characterised.

Despite the undoubted power of existing methods, there remain applications where established tools do not perform well and so a need for constant development of new technology exists. For example, of the current techniques that provide biomoleular structural insight, NMR cannot be applied to large protein molecules, while crystallography requires molecules to crystallise and interrogates the cold solid phase rather than physiologically-relevant room temperature solutions, a feature it shares with electron cryo-microscopy. All of these techniques require extensive data analysis following the experiments meaning that structural insight is rarely available in a rapid, responsive manner.

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The focus of this review article is the technique of ultrafast 2D-IR spectroscopy. [1] In this case, 'ultrafast' highlights that the 2D-IR technique discussed is a non-linear optical spectroscopy employing short pulse duration lasers, as opposed to a rapid form of IR absorption experiment that can be extended to two dimensions via correlative analysis of datasets. [2]

Since the first demonstration of the ultrafast 2D-IR method in 1998, [3] it has proved to be an unrivalled tool for studying the ultrafast vibrational dynamics and equilibrium structural fluctuations of biological molecules, solutions and some materials. [4-11] The major advantage that 2D-IR offers the spectroscopist in comparison to IR absorption spectroscopy is the ability to measure molecular structural dynamics with sub picosecond resolution, via a pump-probe style experiment that provides access to processes such as equilibrium structure fluctuations and hydrogen bond dynamics. This time resolution is combined in 2D-IR with frequency dispersal of both pump and probe events so that the 2D-IR spectrum is a correlation map of excitation (pump) and detection (probe) frequency, revealing energy transfer and coupling between vibrational modes. These processes both give rise to off-diagonal peaks in the 2D-IR spectrum similar to those detected in 2D-NMR spectroscopy, bringing additional information on spatial proximity of functional groups and molecular structure through vibrational modes (as opposed to the nuclear spins measured by NMR).

Of relevance to the analytical science community, this ability to measure vibrational couplings and dynamics means that each molecule produces a unique two dimensional pattern of peaks. As well as being highly sensitive to small changes in biomolecular structure in a way that IR absorption cannot match, for example upon ligand binding to proteins or DNA, [12-15], these patterns can be diagnostic of molecules in complex mixtures. These strengths create the potential for 2D-IR to be applied analytically, but this has only been realised by recent developments in ultrafast laser technology and mid-IR pulse

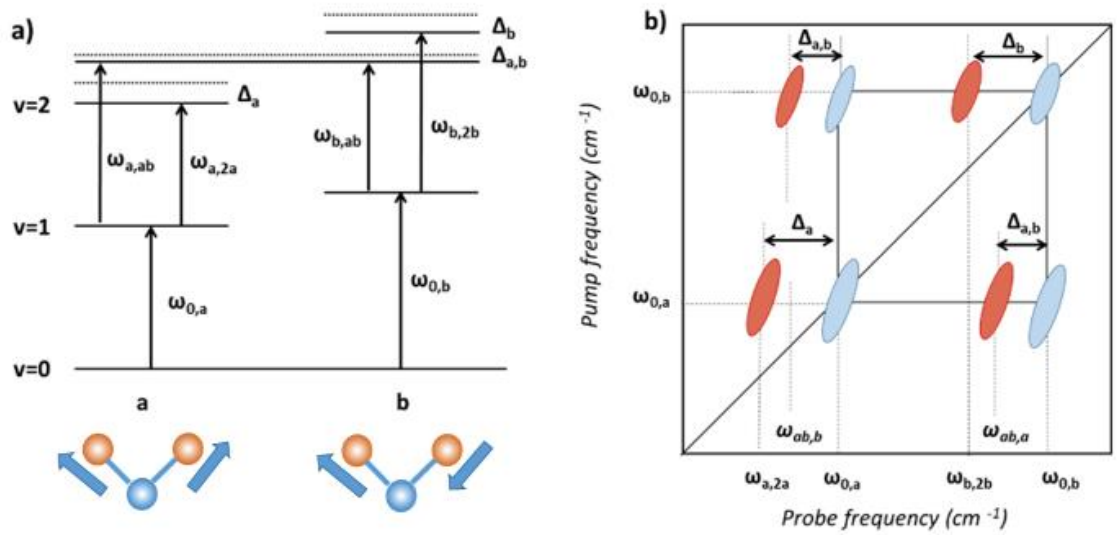

Figure 1: a) Energy level diagram of a model molecular system with two coupled vibrational modes. b) Schematic 2D-IR spectrum arising from the energy levels shown in a). $\omega_{x,y}$ indicate transitions between initial (x) and final (y) vibrational levels. Δ indicates anharmonic shifts of modes relative to a harmonic oscillator model.

Shaping, along with developments in data handling and analysis, that have allowed rapid scanning 2D-IR instruments capable of recording a 2D-IR spectrum in a matter of seconds to be created. [16-20]

The powerful combination of spectroscopic understanding and technological development means that there is genuine promise for 2D-IR to step out of the high-end laser laboratory. This translational process is common to all of the established analytical techniques mentioned above. Each began with a novel physical measurement made in a state-of-the-art research laboratory leading to a period of technique development before the translation into usable technology was achieved. In hindsight, it is easy to forget that a process that can be written in just a few sentences can require substantial amounts of time to achieve. For example, the first article on the characterisation of nuclear magnetic moments was published in 1924, but the first commercial NMR spectrometer did not arrive until 1952, while the now commonplace Fourier Transform technology would take a further 17 years to appear. [21]

With this in mind, we show that the first tentative steps along this pathway have been taken by 2D-IR. We review the progress made in applying 2D-IR to a range of problems, from mixture analysis to biomedically-relevant measurements and high throughput screening. To inform this however, we begin with a brief description of the 2D-IR experiment, showing the key features of the spectroscopy and the technology required for a state of the art 2D-IR spectrometer.

2D-IR Spectroscopy

The development of 2D-IR spectroscopy has taken place over more than two decades and the subject has been reviewed many times previously. [1, 4-11] Rather than repeat that, we will pursue our analytical focus by concentrating on the most up-to-date technology needed for fast 2D-IR data acquisition. We will however begin with a general description of the 2D-IR technique and a discussion of an example spectrum and assignment before progressing to a discussion of the experimental approaches used to acquire 2D-IR data.

2D-IR spectrum of coupled vibrational modes

One of the main strengths of 2D-IR spectroscopy lies in the detection of off-diagonal peaks, which reveal vibrational couplings and energy transfer processes between vibrational modes. To show how such processes are manifest in the 2D-IR spectrum of a simple functional group, we will consider a model system with two vibrationally coupled modes (Fig.1).

The AB_2 molecule shown in Fig.1(a) features symmetric and antisymmetric vibrational modes (denoted **a** and **b** in Fig.1(a)). If we assume that the antisymmetric vibration occurs at a higher frequency than the symmetric mode then we can construct the energy level diagram shown in Fig.1(a), which shows the first two excited vibrational states (v=1 and v=2) of each of the two modes.

The essence of vibrational coupling is that that excitation of one vibrational mode of the pair influences, or changes the frequency of the other mode. In general, coupling occurs when the functional groups involved in the modes are separated by a small number of bonds (<4) within a molecule or are in close spatial proximity such that they interact via a through-space electrostatic mechanism. Similarly, functional groups that are linked via an inter- or intra-molecular interaction such as H-bonding will also be vibrationally coupled. The latter mechanism is particularly important in biomolecular secondary structure.

The implications of vibrational coupling for the energy level diagram (Fig.1(a)) is the presence of a shared ground state for the two modes and the presence of an anharmonically-shifted combination band, featuring one quantum of vibrational energy in each of modes **a** and **b** at an energy lower than the sum of the individual v=0-1 transitions, $\hbar\omega_{0,ab} < \hbar(\omega_{0,a} + \omega_{0,b})$.

A 2D-IR spectroscopy experiment can be considered to be a dispersed frequency pump-probe experiment, in which each vibrational mode of the molecule is excited in turn and a pump-

probe spectrum measured to reveal couplings and energy transfer processes between the excited mode and all others. The pump and probe events are caused by the arrival in the sample of ultrashort duration (~50 fs) laser pulses tuned to the frequency of interest. Effectively, this provides a map of the interactions and chemical and spatial relationships of the vibrational modes and so functional groups of a molecule, adding another layer of detail to the frequencies and intensities that are obtained from IR absorption measurements.

The schematic 2D-IR spectrum (Fig.1(b)) of the system shown in Fig.1(a) can be constructed by first setting the frequency of the 2D-IR pump pulse to be resonant with the v=0-1 transition of mode **a** ($\omega_{0,a}$)**.** In a pump-probe experiment, dynamic information can be obtained by varying the time delay between the pump and probe pulses (called the waiting time T_w), but we begin with a T_w value close to 0 ps.

With a pump frequency of $\omega_{0,a}$ a pair of peaks is observed near the spectrum diagonal. One of these is a negative peak (blue in Fig.1(b)) located at a probe frequency of $\omega_{0,a}$ and the second is a positive peak (red) shifted to lower probe frequency. These are assigned to the $\omega_{0,a}$ and $\omega_{a,2a}$ transitions of mode **a** respectively. The separation of the peaks along the probe frequency axis is due to the anharmonicity of the **a** mode (Δ_a). The peaks arise from a pump-induced transfer of population from the vibrational ground state to v=1 of mode **a**. The 2D-IR measurement can be thought of as a differential absorption measurement, where the absorption of the probe is measured in the presence and absence of the pump pulse, and the difference between the two measurements recorded. Thus, population of v=1 of mode **a** leads to a reduction in absorption of the probe at $\omega_{0,a}$ and so a negative peak (bleach) while the positive peak arises from the new absorption at the v=1-2 transition frequency of mode **a** ($\omega_{a,2a}$) made possible by the pump.

It can be seen in Fig.1(b) that peaks also appear in the off diagonal region of the spectrum. A negative peak is observed at (pump, probe) = ($\omega_{0,a}$, $\omega_{0,b}$) this arises due to coupling of modes **a** and **b**. The common ground state means that bleaching the ground state absorption of one mode will bleach the other. A positive off-diagonal feature is also observed at ($\omega_{0,a}$ $\omega_{ab,a}$) corresponding to the transition from the first vibrationally excited state of mode **a** to the two quantum combination band featuring excitation of modes **a** and **b**. This pair of off-diagonal peaks are separated by the off-diagonal anharmonic shift, Δ_{ab}, which is the value by which the combination band is lower in energy than the sum of the $\omega_{0,a}$ and $\omega_{0,b}$ transitions, a value which scales with increased coupling of the two modes.

When the pump frequency comes into resonance with $\omega_{0,b}$, another horizontal line of peaks appears that can be assigned analogously to those described for a pump frequency of $\omega_{0,a}$, leading to a near-square arrangement of pairs of positive and negative peaks linking the two vibrational modes.

Thus the 2D-IR spectrum of a pair of coupled peaks features two peaks on the diagonal, arising from the v=0-1 transitions of the modes, as would be observed in an IR absorption experiment, but off-diagonal peaks linking the diagonal modes indicate the presence of coupling. These off-diagonal peaks provide structural information on the two coupled modes in addition to indicating the coupling strength. The relative amplitudes of the diagonal and off-diagonal peak pairs as a function of input pulse polarization can be used to extract the angle between the transition dipole moments of the two modes. [1, 22]

Increasing the value of T_w in a 2D-IR experiment causes the diagonal features to reduce in intensity as the v=1 level populations relax. In addition, energy (or vibrational population) transfer may be observed if excitation of v=1 for one of mode **a** or **b** leads to a transfer of that vibrational population to the other mode, e.g. from v=1 of mode **a** to v=1 of **b**. This can be visualised as follows: at a pump frequency of $\omega_{0,a}$ population of the v=1 level of mode **a** is created by the pump pulse. If this population is transferred to the v=1 mode of mode **b** then this creates the scope for the probe pulse to excite the transition $\omega_{b,2b}$. This was not possible without the energy transfer process and leads to the growth of a new peak in the 2D-IR spectrum at (pump, probe) = ($\omega_{0,a}$, $\omega_{2b,b}$).[60,61] In reality, this peak will appear very close to the peak arising from the transition from v=1 of mode **a** to the combination band ($\omega_{a,ab}$), making it hard to detect clearly. Indeed, the small peak separations and low frequency resolution of a typical 2D-IR experiment (2-4 cm^{-1}) often means that energy transfer processes are detected as a growth in amplitude of the off-diagonal peak relative to the diagonal peaks.

Any polyatomic molecule gives rise to a characteristic set of vibrational modes, however, these can overlap in frequency and it is difficult to separate peaks from components of mixtures using absorption spectroscopy. By contrast, in a 2D-IR spectrum the set of vibrational modes along the diagonal are accompanied by additional off-diagonal peaks arising from higher lying vibrational levels and their interactions. Thus, each molecule produces a near-unique 2D fingerprint. [23] Furthermore, in biological molecules such as proteins and DNA, the formation of secondary structural elements leads to specific coupling patterns, such as between the amide I (C=O stretching) modes of the peptide links in an α-helix or β-sheet that are more characteristic than the amide I band as it appears in the IR absorption spectrum. [24] These patterns will form the basis of the examples of 2D-IR analytical applications discussed below.

Measuring a 2D-IR spectrum

The schematic 2D-IR spectrum in the preceding section was described as a frequency-dispersed pump probe spectrum for simplicity. While it is possible to obtain the spectrum practically in this manner, the so-called double resonance 2D-IR method has been largely replaced by time-domain approaches. [25] These can be thought of as the equivalent of replacing a grating-scanning absorption spectrometer with a Fourier transform instrument. Rather than scan the frequency of the 2D-IR pump pulse for a fixed value of T_w to acquire a spectrum, as in double resonance experiments, the pump pulse is instead split into two components with a variable time delay between them.[1, 4-11]

A range of beam geometries have been employed, but, in keeping with our focus on analytical implementations where robustness and simplicity are prioritised, we focus here on the

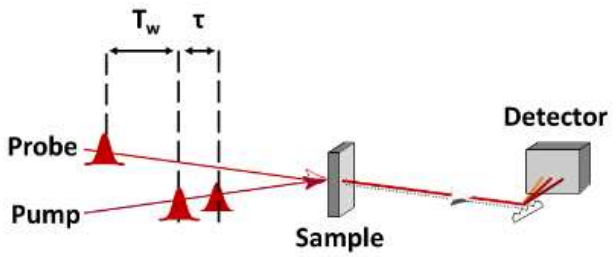

Figure 2: Schematic diagram of a 2D-IR experiment.

most experimentally-straightforward implementation. The experiment takes the form shown in Fig.2. The two pump pulses are arranged collinearly and overlapped spatially with a probe pulse in the sample. The 2D-IR signal arising from the interaction of the three pulses on the sample is formally a three pulse photon echo and is emitted in the direction of the probe beam. The signal and residual probe light are directed into a grating spectrometer and detected with an array detector, creating the 'probe frequency' axis of the 2D-IR spectrum. This is collected over a range of pump-pump delay times, denoted τ, for fixed T_w value. Fourier transformation along τ then provides the pump frequency axis of the spectrum. Samples are typically held between two IR transmissive windows with a path length of 6-50 μm and contain around 30 μL of solution. For a typical protein, concentrations lie in the sub mM range.

A range of methods exist to create and control the pump pulse pair. This can be achieved through an optical interferometer, but mid-IR pulse shaping offers the significant advantages of no moving parts, and hence higher scanning speed, alongside precise control of the pulse timings. The latter is particularly beneficial for analytical applications because it removes uncertainties in the relative timings of the two pump pulses during application of the Fourier Transform, reducing the potential for lineshape distortion in the final spectrum. [18, 19]

We return to a discussion of recent advances in instrumentation below, following a summary of scientific advances in the application of 2D-IR to analytical problems.

Analytical Applications

2D-IR spectroscopy has been widely applied to study the vibrational and molecular dynamics of a range of systems. These have been reviewed previously and lie outside of the scope of this article. [1, 4-11] Here, we focus upon analytical applications of 2D-IR. Prior to discussing specific studies, it is instructive to consider what is meant by analytical applications. The primary goal of the ultrafast spectroscopist is the understanding of the time evolution of quantum states of a molecular system, whether in terms of coupling, coherent processes, energy transfer mechanisms or as a tool to understand intermolecular interactions, for example in the solution phase or of large biological molecules. These studies provide the valuable detailed 2D-IR spectroscopic understanding from which analytical applications can build. For the purposes of this article, we define an analytical application of 2D-IR as one where the primary goal is to make a measurement that informs another aspect of a scientific project. For example, providing information to guide a synthetic chemistry programme seeking to design a small molecule ligand to bind to a particular target.

In such an experiment, 2D-IR would be used to determine the salient structural details of the binding interaction before a next-generation candidate ligand is produced, rather than necessarily seeking to extract highly detailed information about the quantum mechanics or dynamics of the system. This means that the manner of the 2D-IR experiment is different. The key to an analytical measurement is speed, ease of access to key information and, crucially, the ability to cross compare results from a range of samples, for example screening a range of ligand candidates against the target molecule. The latter in particular brings challenges for analytical 2D-IR in terms of measurement to measurement repeatability, where sample cells, laser powers and alignments can fluctuate between, and sometimes during measurements. We return to these challenges in the outlook section below.

In the following sections we introduce a series of measurements that make steps towards analytical applications of 2D-IR, focussing on measurements of protein structure, studies of ligand binding to biomolecular systems with a view to pharmaceutical applications and finally exploration of the potential for 2D-IR spectroscopy to be applied in a biomedical context.

Protein structure determination

Given the close correlation between structure and function, protein identification and protein structure determination are important goals for analytical methodologies. Structural biology is currently dominated by crystallography, NMR and increasingly electron cryo-microscopy techniques. Protein identification in terms of amino acid sequence is currently the preserve of mass spectrometry. Although unable to match the atomistic resolution of crystallography, the ability to operate in solution at room temperature and to deliver information on structure changes and ultrafast structural dynamics sets 2D-IR apart from these methods, meaning that it can provide useful complementary information. In addition, the ability of 2D-IR to measure spectra in just a few minutes per sample means that there may be scope for 2D-IR methods to act as high throughput triage method, identifying samples for further deeper investigation, saving both time and resources. [20] In this respect it is important to note that the majority of the applications of 2D-IR spectroscopy highlighted here were performed in a label-free manner, without the need for implantation of vibrational labels or other sample manipulation. This is an important consideration when attempting to produce results quickly and with no expensive or time consuming sample preparation requirements.

2D-IR has also demonstrated the ability to provide dynamic insight. [1, 4-11] As drug design turns towards therapeutic molecules that target dynamic protein structures, such time resolved insight may become a powerful tool. [26] In such cases, the ability to measure changes in structure, often affecting a small fraction of the overall protein

molecule, may become important. With this in mind, several 2D-IR studies have assessed the ability to identify, categorise and measure protein structures. The spectroscopy is based around details of the 2D amide I band of proteins, which are intimately linked to the vibrational coupling arising from H-bonding patterns inherent in secondary structures. The off-diagonal structure of the amide I band in a 2D-IR spectrum has been used to identify β-sheets[27, 28] and α-helical structures. [29] These studies formed the basis for studies showing that 2D-IR can quantify protein secondary structure content in a set of 16 proteins of varying degrees of β-sheet and α-helix content. [24]

In terms of measuring small changes in structure, it has been shown that the ratio of the magnitude of the 2D-IR amide I band to that from IR absorption spectroscopy provides the ability to measure the amide I transition dipole moment. [30] The latter is closely dependent upon the extent of coupling within a macromolecular structure and allows observations affecting the integrity of secondary structural elements. [31]

When developing analytical technologies, it is important to benchmark new methods against existing tools. In terms of protein secondary structure determination, circular dichroism (CD) sets the standard for such measurements. It is thus important that 2D-IR has been shown to provide similar accuracy in terms of quantification of secondary structure, with a study of the Ca^{2+} binding transition of calmodulin showing that 2D-IR spectroscopy is sensitive to a secondary structure change affecting just seven residues of the 150 residue protein. [26]

Thus far, we have focused on 2D-IR experiments using broadband, short duration infrared pulses as the source of the excitation and detection pulses. However, a second, less widely used 2D-IR method called electron-vibration-vibration (EVV) 2D-IR has been applied to protein structure determination. [32] The 2D-IR spectrum produced by the EVV method is broadly similar to that from the all-IR 2D-IR techniques discussed so far in that it features a correlation of excitation and detection frequencies and provides a map of coupled vibrational modes. The major differences between the two methods lie in the fact that EVV uses two narrow bandwidth IR pulses to excite specific vibrational modes and that one of those IR pulses is often tuned to an overtone or combination band rather than a fundamental transition. Following these two IR excitations, the signal is 'read out' using a visible wavelength pulse which excites a Raman transition. The latter is advantageous in that it allows visible wavelength detection to be employed, with CCD cameras offering significant improvements in sensitivity over the HgCdTe technology that is the current state of the art for IR pulse detection. EVV methods have been used to show that specific patterns of off-diagonal peaks can be assigned to amino acids and that detection of levels of certain key amino acids allow identification of proteins. [33] This encouraged discussion of 'optical proteomics' methods in which EVV spectroscopy provides a basis for determination of amino acid content. [34] For example it has been suggested that monitoring the ratios of five amino acids would allow identification of 44% of the (unmodified) human proteome, while extending this to 6–9 amino acids could raise this number to 90%. The EVV method also allows very specific localised insight into protein structures. For example, it has been reported that EVV can be used to quantify nitration of tyrosine residues, opening the door to studies of post-translational modifications. [35]

Ligand binding

A growing body of evidence suggests that a particular area of strength for 2D-IR spectroscopy is the development of streamlined platform for structure-based evaluation of biomolecule-ligand binding.

The ability of 2D-IR to evaluate small molecule based inhibition was demonstrated in a study of the ability of sulfonated triphenyl methane derivative acid fuchsin to inhibit amyloid formation by islet amyloid polypeptide. [36] In this case, 2D-IR was employed alongside a suite of biophysical methods to characterise the behaviour of the inhibitor molecule. This study in particular exemplifies the development time needed for analytical applications of 2D-IR to come to the fore, building as it did on several studies of amyloid spectroscopy, structure and formation mechanisms, many of which employed site specific labelling in order to reveal molecular and atomistic details of the amyloid formation process. [19, 37, 38] This is likely to be a strength of 2D-IR as it progresses towards analytical applications: while a straightforward triage-style measurement, rapid test or screening experiment can be designed and employed, the wealth of information from the spectroscopy will always be present in the measured data, allowing detailed post-experiment analysis.

Applications of label-free 2D-IR to drug binding studies have shown a sensitivity to small changes in protein structure and dynamics upon binding. A study of inhibitor binding to the InhA enzyme, a component of the fatty acid synthase pathway responsible for maintaining cell wall integrity in *Mycobacterium tuberculosis* and a validated drug target, showed that changes in the off-diagonal region of the amide I 2D-IR band correlated with drug binding and enzyme inhibition. [13] In particular, this component of the 2D-IR amide I lineshape was less strongly affected in mutations of InhA found in drug resistant forms of the bacterium. Combining spectroscopy with modelling, it was demonstrated that the 2D-IR signal was responding to changes in dynamics of the protein backbone caused by drug binding, a particularly subtle effect given that no large scale structure change accompanies binding. [13] This shows the ability of 2D-IR to go beyond the structure function relationship, providing new insight. Once again, this was a detailed spectroscopic study, but it can be seen that the 2D-IR signature of 'effective inhibition' provides the basis for a screening study of new prospective ligands and hence a route by which 2D-IR can contribute to the drug design process. In particular, the fact that the effectiveness of a ligand is manifest as a dynamic change rather than a physical, structural one means that established analytical techniques will be less effective than 2D-IR.

2D-IR methods have also been applied to the quantification of ligand binding. Traditionally, the preserve of isothermal titration calorimetry, it was shown that peptides labelled with the non-natural amino acid azidohomoalanine could be observed binding to a PDZ2

domain from the protein tyrosine phosphatase 1E.[14] The experiments showed that it was possible to obtain a dissociation constant for the peptide in the micromolar range. It is relevant that the addition of vibrational label groups (azide-modified amino acids) to the system were inherent in the measurement, however, the authors point out that similar methods could in principle be applied to spectroscopic signatures from intrinsic functional groups and the measurements were accompanied by work to reduce the detection limit for 2D-IR to around 100 µOD, which will bring weakly absorbing species within the detection limit.

EVV-2D-IR methods have also been applied to studies of protein-ligand binding. Using a label-free approach the binding of drug molecule SU5402 to its FGFR1 kinase target protein was studied. [39] A further difference between EVV and the more widely applied 2D-IR method can be seen in the information rich spectra produced by EVV (Fig.3). In this kinase study ~200 detectable cross-peaks were observed in the spectral region 1250–1750 cm^{-1}/2600–3400 cm^{-1} and a further 63 peaks were observed upon ligand binding. The authors showed that, of the 63 new peaks, six could be definitively assigned to protein–drug interactions, while the remainder were due to vibrational coupling within the drug itself. Combining experiment with quantum mechanical calculations identified one of the six peaks to a known interaction between the drug and a backbone carbonyl group which forms part of the binding site. As modes assigned to intramolecular coupling peaks of the drug molecule did not change substantially upon binding, it was concluded that the structure of the drug in the target binding site is essentially identical to that when it is not bound.

This study nicely highlights the complementarity of the EVV 2D-IR method with the all-IR approach. By producing large numbers of peaks that can be isolated to specific parts of the drug and protein structure, EVV generates a very detailed local picture of the drug protein interaction. By contrast, when applied without the use of site specific labelling methods, the all-IR 2D-IR method focuses upon more global changes in the protein structure or dynamics. Such a combination of local and global insight could prove extremely powerful in drug design studies, especially when allosteric mechanisms and long-range communication effects in biomolecules are at work. Of further relevance, it has been shown that EVV methods can be used to measure molecular geometries in non-covalent interactions. [32] Though not trivial and requiring significant computational effort, such measurements are a powerful addition to the 2D-IR suite of techniques.

A highly desirable feature in an analytical tool is the ability to keep pace with the project that it is supporting. Returning to the hypothetical synthetic chemistry project discussed earlier or speculating about a role for 2D-IR in commercial drug design processes, this would require the ability to collect and process spectra quickly. To this end, a pilot study showed that 2D-IR spectroscopy could be used to study ligand binding to double-stranded DNA, collecting data on 12 DNA sequences with and without a ligand at a range of T_w values. [20] The dataset consisted of over 2000 individual 2D-IR spectra. In such a case, analysing each spectrum peak by peak is neither desirable nor practicable. Instead analysis of variance-principal component analysis (ANOVA-PCA) was

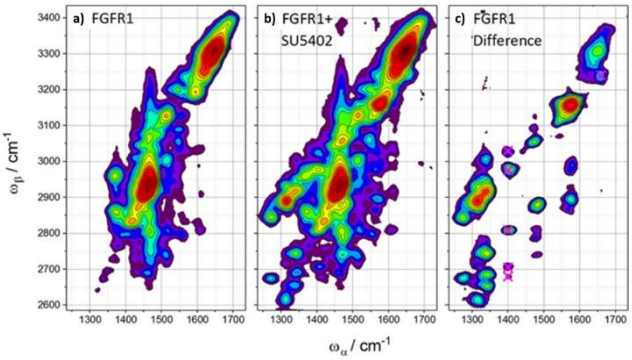

Figure 3: Experimental EVV 2DIR spectra of (a) FGFR1 kinase protein, (b) FGFR1 plus the SU5402 drug molecule and (c) difference spectrum of FGFR1 + SU5402 and FGFR1 taken from Ref [39]. The difference spectrum from FGFR1 (c) was found to contain an additional 6 peaks in comparison to a difference spectrum of SU5402 bound in a nonspecific state to bovine serum albumin. [39] These were ascribed as binding dependent peaks. The frequency locations of these 6 binding-dependent peaks are marked using pink crosses. Adapted with permission from *J Phys Chem B*, 2019, 123, 3598-3606. Copyright (2019) American Chemical Society.

used. This method was developed for mass spectrometry analysis and seeks to determine sources of variance in the data based upon experimental factors (*e.g.* sequence, ligand, T_w) that were designed into the dataset at the outset (Fig.4(a)). It was demonstrated that ANOVA-PCA was able to accurately extract the base content of the DNA sequence, detect the presence of a ligand and to sort the sequence-ligand combinations into an order that correlated strongly with independent measurements of ligand binding strength. In doing so it was possible to extract the spectroscopic signatures of DNA base pairs (Fig.4(b)) and of strong, selective binding of the ligand. [20] The result of this study is two-fold, firstly showing that a high throughput screening style experiment is plausible with 2D-IR and secondly showing the sensitivity of the label free spectrum to subtle effects of ligand binding. The aim of the work was to understand the impact of binding to DNA, but this shows the potential for 2D-IR to perform the reverse role, screening a series of ligand candidates to determine those which deliver the required impact upon DNA, i.e. which give strong and specific binding. This is traditionally analysed by biochemical techniques such as DNA footprinting or UV melting experiments, both of which take substantially longer to acquire data and require more ligand material to be produced.

Biomedical applications

Given the relatively early stage of development of 2D-IR methods, applications to biomedical problems are few in number, though two studies in particular show the potential for future development.

A pair of papers reported the application of 2D-IR spectroscopy to study lens tissue taken from the eyes of porcine and human subjects with and without cataracts. [40, 41] The samples revealed that amyloid β-sheet secondary structure was detected in cataract-containing lenses. Cataracts are believed to be formed via aggregation of crystalline proteins and 2D-IR was able to identify the presence of β -sheet structures that had been previously associated with amyloid secondary structure.

Interestingly, the 2D-IR spectroscopic detection was possible even under conditions when the amyloid fibres were too short for detection with electron microscopy (TEM). Furthermore, as no amyloid structures were found in lenses from juveniles, which provided a control for the study, but lenses from more mature subjects with no diagnosed cataracts did contain amyloid, the authors proposed that amyloid structures begin forming before diagnosis, establishing a link between amyloid structure and disease pathology. Although clearly at an early stage, this study establishes a role for 2D-IR spectroscopy in providing molecular insight into what is a common biomedical problem and shows how it could, in future, play a role in pharmaceutical studies seeking new therapeutics.

Although studies of cataract samples were carried out post-mortem, a recent study has shown that 2D-IR spectroscopy could be applied to the molecular analysis of proteins in blood serum taken as part of a routine medical procedure. [42] Spectroscopic interrogation of biofluids is attractive as a label-free, minimally-invasive screening technology.[43] Biofluids, such as blood serum are generally easily obtained, with minimal patient discomfort, and they provide access to a large amount of potentially diagnostic chemical information, reporting as they do on metabolism.[44, 45] Current technologies use antibody assays to enhance the signal associated with a target biomolecule, but this requires specific antibodies for proteins of interest to be available in addition to not insignificant levels of sample preparation. Perhaps more importantly, disease states are complex and so single-metabolite detection may be inferior to a broad biomolecular fingerprint of metabolic function, not least as an early warning of deteriorating patient health. [44-46]

The protein content of blood serum represents an ideal substrate for holistic analysis and is tempting because the amide I band is highly sensitive to protein secondary structure. [24] The problem remains however that, under physiological conditions strong, overlapping water absorptions block observation of the amide I band. In all of the studies reviewed so far (except those using EVV methods) the use of deuterated solutions was used to shift the water bending vibration down in frequency and away from the amide I protein band. However, deuteration is impractical for biomedical or commercial applications of protein IR spectroscopy and raises fundamental questions regarding the impact of increased mass on protein dynamics.

It was demonstrated that 2D-IR spectroscopy can avoid this problem because, in contrast to IR absorption experiments, the 2D-IR amide I signature of proteins dominates that of water even at sub-millimolar protein concentrations. [42] This important result means that 2D-IR can be used to study the amide I band of protein containing samples without the need for expensive, time consuming deuteration of solvents. Equine blood serum was employed as a test system and the unique link between protein secondary structure and the 2D-IR amide I lineshape enabled differentiation of signals due to albumin and globulin protein fractions in serum and quantitative measurements of the biomedically-important albumin to globulin ratio (AGR) with an accuracy of ±4% across a clinically-relevant range. In addition, 2D-IR spectroscopy was shown to be capable of differentiating the structurally similar globulin proteins IgG, IgA and IgM using subtle changes in the serum amide I 2D-IR band. In

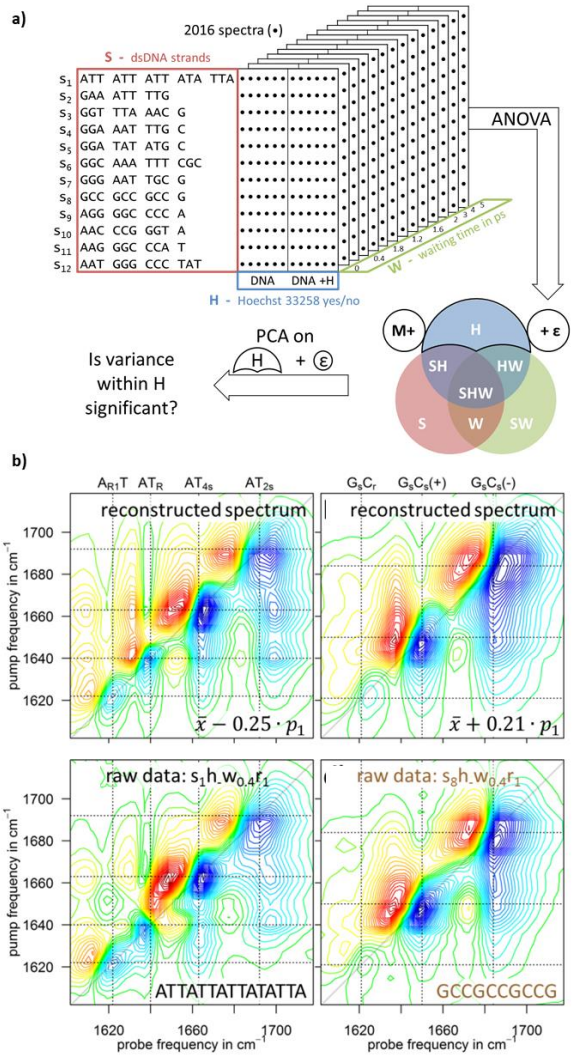

Figure 4: a) Schematic representation of the 2D-IR screening experiment and ANOVA-PCA data analysis methodology reported in in Ref [20]. The diagram summarises how the matrix of 2D-IR spectra can be separated according to key experimental factors such as DNA base sequence, 'S', whether a ligand is present, 'H', or the waiting time of the experiment, 'W' by the ANOVA method. b) Comparison of the spectra of AT (left column) and GC (right column) base pairs DNA obtained from experiments (lower row) and from the ANOVA-PCA analysis (upper row). The agreement is excellent and shows the validity of the screening analysis method. [20]

conjunction with high throughput screening demonstrations discussed in the preceding sections, this article thus motivates new directions in which 2D-IR could be employed as a straightforward spectroscopic approach to measuring levels of serum proteins that are currently only accessible via biomedical laboratory testing.

Instrumentation

It is clear from the progress made thus far that 2D-IR applications are advancing in line with developments in the available technology, both of lasers and associated optomechanical equipment. We have already touched on the significant step forwards brought by mid-IR pulse shaping

technology, both in terms of scan rate and streamlined data processing. [18, 19] A second significant development has been the advent of high pulse repetition rate laser systems. [16, 17] When the first 2D-IR studies were carried out, typical laser systems produced one laser pulse per millisecond, a repetition rate of 1 kHz. This meant that one thousand laser shots per second could be measured. With the required level of laser shot averaging and scanning of time delays using optical delay lines, spectrum acquisition times were on the order of hours. Improvements in laser technology have led to pulse repetition rates of 10 kHz and most recently 100 kHz. With the further improvements brought by pulse shaping, this means that a good quality 2D-IR spectrum can be acquired in as little as 10-20 secs. [20]

While high repetition rate lasers represent the current state of the art, significant steps have also been made to accelerate scanning rates with lower repetition rate systems. The aim in each case being to increase the number of spectrum averages that can be obtained within a particular recording time or to limit the effects of longer term laser drift.[47-49]

Increasing spectral scanning rates means that the rate determining step in a measurement becomes the time for changing the sample and this has motivated developments of microfluidic based tools for flowing the sample through the area of laser beam overlap. [50] Alternative sample delivery strategies include the use of hydrated gel spots for EVV 2D-IR studies, [39] but it is clear that sample handling will become a focus for development as the technique progresses.

One final promising direction comes from studies showing that 2D-IR can be coupled to a mid-IR microscope platform to enable 2D-IR imaging. [51, 52] Although very much proof of concept at this stage, these studies open to door to tissue imaging with the high levels of spectral information available from 2D-IR. A challenge to such applications will come from the spatial resolution possible with mid-IR wavelength beams, but for applications in which the off-diagonal region of the 2D-IR spectrum may provide valuable molecular information, 2D-IR imaging may find a niche.

Summary and outlook

The work highlighted in the preceding sections shows that 2D-IR possesses considerable potential as an analytical tool. However, the translation of this powerful spectroscopic method to a more applied setting faces considerable barriers. Perhaps the most significant of these lies in the equipment and expertise needed to produce a 2D-IR spectrum and analyse the results. State-of-the-art ultrafast laser instrumentation is expensive, while associated equipment for controlling and detecting the pulses adds further non-trivial expense. The expertise required to establish maintain and operate a 2D-IR spectrometer is highly specialised and so a cost-effective, turn-key system that can be operated by a non-expert is not a realistic possibility in the near term. That said, specialist facilities do exist and so a fee for service type arrangement or a centralised facility handling samples remotely is a plausible alternative. In the latter case, the one-off set-up costs and downstream operating costs will be more than compensated by high throughput sample handling, potentially leading to relatively low cost per sample operation. This does not preclude further instrumentation development by laser or other equipment manufacturers, which may lead to more hands-free instruments in due course.

2D-IR also faces challenges relating to data analysis and the ability of non-expert users to extract meaningful insight from information-rich data. This problem is harder to overcome, though as analytical applications evolve, methods such as machine learning or image recognition may become applicable. Care is needed in the use of such methods that the information retrieved is firmly grounded in the underlying spectroscopy, but with the ability to record large datasets, cross comparison of spectra over a larger sample space will be expected to become very informative to a community in which subtle spectral details can be dismissed as an artefact or overlooked in one-sample studies. Uptake and development by the 2D-IR community will then drive translation of these advanced tools.

Sensitivity is a perennial challenge to all analytical tools, there will always be a lower concentration limit, below which meaningful data is hard or impossible to recover. 2D-IR spectroscopy is not a high-sensitivity analytical tool, though advances in technology and methodology are leading to ever lower concentrations of biomolecule samples being accessible. In this, the sample requirements of 2D-IR are comparable to those of NMR, but improvements in detector technology and laser capability may see this capability extended.

In addition to improvements to the hardware of a 2D-IR experiment, significant steps have been taken to improve sensitivity via the sample. Examples include attempts to use surface enhancement[53, 54] and the use of plasmonic surface structures [55, 56] including recent demonstrations of an immunological sensor with monolayer sensitivity.

Other areas where advances can be expected in the near term include sample handling and the use of more automated technology to exploit fast data acquisition times. The advent of microfluidics in 2D-IR experiments has already been reported and this can be expected to expand. [50]

It is clear that advances in all of these areas, from hardware, through to measurement protocols and physical or sample presentation methods will all play a significant role in helping 2D-IR to reach ever lower molecular detection levels and in turn will enable high throughput methods to reach their full potential.

One area of 2D-IR spectroscopy that has not been dealt with in detail in this review is the ability of the method to extract dynamical information. The details of the approach have been reviewed elsewhere, [1, 4-11] but the lineshapes of 2D-IR spectra evolve with T_w in ways that reveal energy transfer and local chemical exchange processes. These have been extremely powerful in revealing the fundamental dynamic processes occurring in biological molecules and even as molecular rulers, [57] but have yet to be applied in a truly analytical context. However, the potential future use of dynamic signatures as analytical markers and indeed, the ability to perform analysis based upon ultrafast dynamics would be an area in which 2D-IR would be unmatched and this could be a very exciting future direction.

In summary, although challenges exist, the benefits from the additional information and insight of 2D-IR methods, including the ability to 'see past' the water absorption problem that has restricted the use of IR absorption in biological, biomedical or pharmaceutical arenas, should lead to the continued development of what could prove to be a powerful new analytical technology.

Conflicts of interest

There are no conflicts to declare.

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